Video-rate two-photon microscopy of cortical hemodynamics in-vivo

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Abstract: A video-rate two-photon microscopy system was constructed for optimized in-vivo imaging of functional activation and hemodynamics in rat brain. The system has been used to explore the vascular mechanisms underlying functional responses and baseline oscillations.

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1. Introduction

Two-photon microscopy has rapidly become a valuable tool for high-resolution imaging in-vivo. The ability to resolve individual red blood cells circulating through the vasculature of the rat brain to depths of >500 microns following intra-venous (IV) injection of fluorescein presents new opportunities to explore the mechanisms of hemodynamic activation in the rat cortex [1]. The fact that additional dyes or other markers can also be introduced with fluorescein to explore neuronal [2] and glial activity alongside vascular responses means that two-photon microscopy is rapidly becoming an unsurpassed imaging modality for neuroscience research.

We have recently developed a custom two-photon microscope system at MGH. The microscope was specifically designed to provide a highly versatile and flexible platform for in-vivo, video-rate, wide-field two-photon microscopy. The system can currently achieve bi-directional line-scan rates of ~3300 lines per second, with lateral resolution of <1 µm to depths of >450 µm in-vivo. The system has been used to image cortical hemodynamics in rats undergoing electrical forepaw stimulation. The steady state and stimulus-induced hemodynamics within the different vascular compartments (arteries, arterioles, capillaries, venules and veins) can be readily explored in terms of red blood cell speed, flow and vessel diameter. Via spatiotemporal analysis of video-rate, wide-field images of the brain's vasculature during stimulus and baseline conditions, we have explored how the behavior of individual vessels corresponds to the ensemble hemodynamic functional response commonly observed via exposed cortex optical imaging, non-invasive brain near infra-red spectroscopy and functional magnetic resonance imaging [3, 4].

2. Instrument design

The custom two-photon microscope uses a Ti:Sapphire laser (Mai-Tai, Spectra Physics) tunable from 710nm to 920nm. The beam passes through a polarization-based variable attenuator, through a conditioning telescope and up a periscope to vertically mounted galvanometer mirrors (6215HB, Cambridge Technology). These mirrors steer the beam through a 1” diameter, achromatic, 50mm focal length scan lens followed by a near infrared (NIR) corrected trinocular and tube lens assembly (U-TR30NIR, Olympus). The resulting collimated beam passes through a 700nm long pass dichroic beamsplitter and into an infinity and NIR corrected objective (e.g. Olympus XLUMPlanFl 20x/0.95W). The emitted two-photon fluorescence is reflected off the dichroic beamsplitter, passes through two 700nm short pass filters and a second dichroic beamsplitter (550nm long pass). Two photomultiplier tubes (R3896, Hamamatsu) are positioned around the second dichroic beamsplitter to detect the spectrally discriminated emission.

For precise control of the focal plane the objective is mounted on a computer-controlled fine Z-adjustment stage (M-112.1DG, PI). This stage is mounted on a Nikon Modular Focusing Unit (MBD64010) to allow coarse adjustment of the objective position and enable manual focusing during visual observation through the trinocular eyepieces. The objective is suspended in free-space, providing unrestricted access to position the animal beneath it. The animal itself is positioned in a stereotaxic frame which sits on top of two orthogonal linear stages (M-126.DG1, PI) which allow for computer control of the XY plane of the field of view. Data acquisition and galvanometer control are synchronized via two National Instruments DAQ cards (PCI-6713 and PCI-6115). The microscope operates with bi-directional scanning.

The microscope is controlled via a custom Matlab Graphical User Interface (GUI) which provides complete automation of all aspects of the microscope, including laser power, stage movement and data acquisition. The system can be operated in several acquisition modes to allow rapid acquisition of stacks, line-scans and wide-field images, with image rotation achieved by modification to the galvanometer driving waveforms.
3. Animal preparation and data acquisition

Rats were surgically prepared via tracheotomy and insertion of arterial and venous femoral catheters under isoflurane anesthesia. The rat was placed in a stereotaxic frame, the scalp retracted and the skull and dura over the somatosensory cortex was removed. Agarose (~1.5% wt/vol) mixed with artificial cerebrospinal fluid (ACSF) [1] was placed between the cortex and a small round glass cover slip, which was then secured rigidly relative to the skull using dental acrylic. The resulting cranial window provided a rigid seal over the exposed cortex, protecting the brain and preventing motion artifacts. Two needles were inserted into the rat’s forepaw and connected to a stimulus isolation unit (A360, WPI). Isoflurane was then replaced with IV alpha-chloralose.

Forepaw stimulus consisted of 4 seconds of 3 µs-width pulses at 3Hz with ~1mA amplitude. Prior to two-photon imaging the rat was positioned under a CCD camera for conventional wide-field optical imaging of the cortex with 580nm light. 30 repetitions of 4 seconds rest, 4 second stimulus, 14 seconds rest were acquired. Data was block-averaged and the activation maps were used to guide selection of regions for two-photon investigation.

The rat was then positioned under the two-photon microscope objective. A ~4.5% wt/vol solution of dextran-conjugated fluorescein (FD2000S, Sigma-Aldrich) was injected IV to provide fluorescence contrast to the blood-plasma. The animal was continually ventilated with 30% oxygen in air. Blood pressure, body temperature and anesthesia level were monitored throughout. All animal procedures were reviewed and approved by the Subcommittee on Research Animal Care at MGH.

The two-photon microscope field of view was positioned over the forepaw-activated region of the cortex identified via the geography of the superficial vessels from the CCD images. The field of view was then further adjusted to locate regions where capillary beds, arterioles and venules could be easily visualized, if possible within the same image. The stimulus cycle was then presented to the forepaw every 22 seconds over 5 stimulus cycles during triggered rapid acquisition of two-photon images of a selected region of the brain.

4. Results

4.1 Multi-scale stimulus response

Figure 1 shows successive imaging of functional activity using conventional optical imaging of the exposed cortex, followed by closer examination using two-photon microscopy. By examining a wide-field two-photon image series, individual vessels can then be analyzed in terms of their diameter changes, changes in their hematocrit (inversely proportional to the intensity of the signal from the vessel / area of the vessel) and the speed of the flow in the vessel. While blood flow can be measured using laser speckle imaging and Laser Doppler, separately measuring the speed of flow and diameter changes can elicit significantly more information about the mechanisms underlying the control of hemodynamic changes. Figure 2 suggests that arterioles actively change their diameters in response to stimulus, whereas veins and venules exhibit almost no diameter changes. This is in contrast to the common assumption in fMRI analysis that CBV changes occur mostly in the venous compartment [5].
4.2 Oscillations in the vascular compartments

In another rat, ‘Vasomotor’ or ‘Mayer Wave’ 0.1Hz oscillations were present, as are commonly observed in lower resolution functional imaging experiments [6, 7]. In order to examine these and other baseline signals in our two-photon images, principal component analysis was used. This allowed the major changes in the images over time to be identified and spatially localized.

Figure 2 shows the first three spatiotemporal principal components (PCs) of a 9 second image series acquired at 22 frames / second with no stimulus. PC1 resembles the mean image as expected. PC2 clearly reveals the 0.1Hz signal, and localizes it to only the edges of the arteriole in the field of view (and not the veins). Further analysis of the vessel diameters, confirms that the arteriole is actively contracting and dilating. A correlation coefficient map also illustrates that the signal is originating from movement of the arteriole walls. PC3 correlates strongly with the arterial blood pressure, showing heart beat and breathing modulations. These are distinct from the 0.1Hz wave and are evenly distributed between the arterioles and veins.

From this preliminary data we hypothesize that the ‘vasomotor’ signals often observed in studies of cerebral hemodynamics are a result of local control of arteriolar diameter, rather than systemic blood pressure variations, i.e. due to Vasomotor activity rather than systemic Mayer waves. We plan to further examine the nature of the heart-rate and breathing-rate vascular dynamics to determine the vascular mechanisms responsible for hemoglobin concentration modulations in the resting brain.

5. Summary

Using a custom-built in-vivo two-photon microscope we have measured in detail the dynamics of the different vascular compartments in the cortex during functional activation. We plan to continue to develop the microscope system to add new functionalities, and to develop the experimental paradigm to include dyes that can elicit information about neuronal and glial electrodynamics and oxygen perfusion dynamics. We will present the basic design of the new system, describe its characterization and present our latest in-vivo results.

6. References